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LC-MS Analysis of Dimethyl Fumarate in Rat plasma with Measurement Uncertainty Estimation

Abstract

Dimethyl fumarate (DMF) is the methyl ester of fumaric acid initially recognized as a very effective hypoxic cell radio sensitizer. Phase III clinical trials found that DMF successfully reduced relapse rate and increased time to progression of disability in multiple sclerosis. Small molecules like dimethyl fumarate pose particular difficulties when analyzing biological samples due to the increased possibility of matrix effects and in this scenario DMF immediately converts to its active metabolite MMF (Monomethyl fumarate) by oral route. It is for this reason that a sensitive liquid chromatography–tandem mass spectrometry method has now been developed for the analysis of DMF and for studying the pharmacokinetic profile in rats. Sample preparation was by rapid protein precipitation with acetonitrile. Analyte separation was achieved on a reversed-phase XTerra MS C18 column (100 x 3.9 mm, 3.5µ) with 0.01M ammonium formate and acetonitrile in gradient mode as the mobile phase at a flow rate of 1.0 mL/min and analyzed by a hybrid triple-quadrupole linear ion trap mass spectrometer in positive electrospray ionization mode for both DMF and MMF. Limits of detection, and quantification were 20 and 50 ng/mL, for DMF and 1 and 10 ng/mL for MMF respectively. Calibration curve showed excellent linearity within the 50–2500 ng/mL range for DMF and 10–500 ng/mL range ($r2 > 0.999$) for MMF. Intra- and inter-day precision defined by coefficient of variation was <10% and accuracy (bias %) was within 90–110%. Measurement uncertainty estimation was 8.6% for DMF and 11.6% for MMF. The method has been successfully used in the analysis of DMF and MMF in rat plasma following its administration to male wistar rats for pharmacokinetic studies.

Keywords: Dimethyl fumarate, Rat plasma, Pharmacokinetics, LC-MS, Measurement of Uncertainty, Monomethyl fumarate

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1. Introduction

Multiple Sclerosis (MS) is an inflammatory condition that damages the myelin of the Central Nervous System and causes neurologic impairment and, frequently, severe disability. It is a common neurological disease with prevalence rate ranging from more than 100 per 100,000 in Northern and Central Europe to 50 per 100,000 in Southern Europe. The aetiology of MS remains unknown. It is generally assumed that MS is mediated by some kind of autoimmune process triggered by an infection and superimposed upon a genetic predisposition. Most patients present with relapsing-remitting multiple sclerosis (RRMS), characterized by unpredictable acute episodes of neurological dysfunction or relapse, followed by variable recovery and periods of clinical stability. Within ten years more than 50% of patients who presented with a relapsing-remitting (RR) form eventually develop sustained deterioration with or without relapses superimposed, i.e. secondary progressive multiple sclerosis (SPMS). Around 15% of patients develop a sustained deterioration of their neurological function from the beginning, i.e. have primary progressive multiple sclerosis (PPMS). About 5% of the patients have a steady progression of clinical neurological damage with superimposed relapses, i.e. progressive relapsing multiple scleroses (PRMS).

Dimethyl fumarate (DMF), known as radio sensitizer [1] is recently approved for treatment of multiple sclerosis. Phase 2B trial of its formulation as BG-12 in RRMS patients showed significant decreases in new gadolinium enhancing lesions, T1 and T2 lesions, and a non-significant decrease in the annualized relapse rate [2] and Phase III clinical trials of DMF successfully increased its usage in MS patients [3]. Different HPLC and GC-MS methods have been published for dimethyl fumarate assay in different types of products like desiccants, antimould sachets, consumer products, leather products, [4-12] etc. Till date, there is no method published by LC-MS for determination of dimethyl fumarate in biological samples as it immediately converts to its metabolite MMF. LC-MS methods have the advantage of a higher sensibility, higher selectivity and higher throughput compared with LC-UV methods. In this paper we focused on developing a single LC-MS method for estimating DMF and MMF in rat plasma samples. Imipramine is selected as internal standard (IS) which is compatible with DMF and MMF without any ion suppression and no interference in extraction procedure. The structural formulae of analytes and IS are represented in Fig. 1.

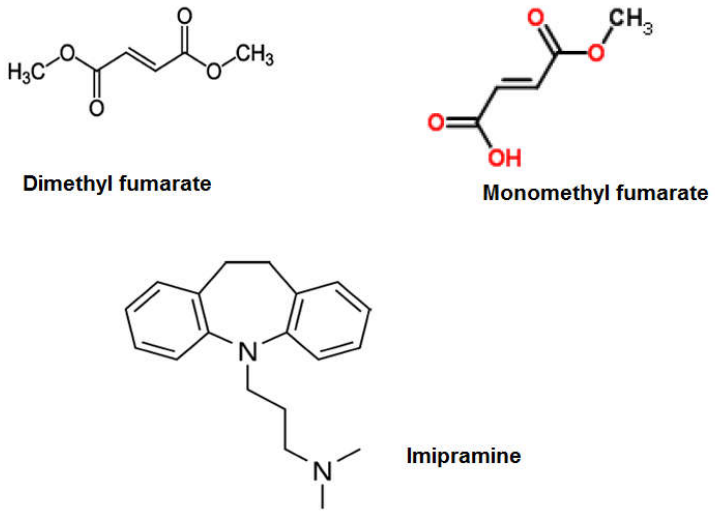

Figure 1: Structural formulae of DMF, MMF and IMP (IS)

Reliable analytical results are needed for correct interpretation of quantitative findings in biopharmaceutics. For this reason, it is important for to provide information on uncertainties of their analytical results. Uncertainty of any measurement is the doubt which exists about the result of a test sample, and it represents a quantitative value of the doubt about the measurement result. Thus, knowledge of the uncertainty is required for correct interpretation of the measurement because such a result is only complete if it is accompanied by a statement of uncertainty in the measurement [13]. Recently, Lee et al. [14] reported on measurement uncertainty (MU) estimation by the bottom-up approach for amphetamine and methamphetamine in urine as a validation data. Measurement of uncertainty estimation is included in this analytical result to re-emphasize the importance of MU estimation of such drugs which effect the livelihood. In this study, a sensitive, rapid, and defensible method by LC–MS was developed with information on MU estimation. The method was critically validated for screening, quantification, and confirmation of the presence of DMF & MMF in a test sample and for application to research on the pharmacokinetic studies.

2. Experimental

Materials

Dimethyl fumarate (DMF) and monomethyl fumarate (MMF) was supplied by Sigma-aldrich. Acetonitrile of MS grade was obtained from Merck, India. Imipramine used as internal standard supplied by Sigma-aldrich. Other chemicals were all of analytical grade and purchased from Merck, India. Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore. Biological matrices were obtained from Vimta Labs (Hyderabad, India) and stored at −20°C until use.

Instrumentation

The LC–MS/MS analysis was carried out in electro spray ionization (ESI) positive mode on a mass spectrometer (API 3000) coupled to a Shimadzu LC system (Model: SIL-HTC) operated with Analyst 1.6.1 software.

Chromatographic and MS parameters

The separation of the analytes was carried out on an XTerra MS C18 (100 mm length x 3.9 mm internal diameter and 3.5 µm particle size) column. Temperature was set to 25°C. The mobile phase composed of 0.01M ammonium formate (A) and acetonitrile (B) (in gradient mode) at a flow rate of 1.0 mL/min for 5 min. Gradient elution performed as: 95% A/5% B hold for 2 min, to 95% B in the next 0.2 min and hold till 4 min and again back to 95%A/5% B in 0.1 min and equilibrated till 5 min. The injection volume was set at 50 µL. The full scan MS and MS/MS spectra of analyte was obtained by direct infusion of the respective sample solution at a concentration of 10 µg/mL solution prepared in methanol. The samples were analyzed using single reaction monitoring (SRM) mode. The spectra were acquired using the following conditions: ion-spray voltage, 5000 V; turbo gas temperature, 250°C; nebulizer gas (compressed air), 55 psi; curtain gas (N2), 20 psi; declustering potential, 80 eV; focusing potential, 200 eV; entrance potential, 10 eV. MS signals were monitored as follows: IS detection (isolation of parent ion m/z= 281 a.m.u.); analytes detection (isolation of parent ion m/z=145.0 a.m.u., for DMF, m/z=131.0 a.m.u., for MMF).

Preparation of calibration samples and quality control samples

Standard stock solutions of DMF and MMF were prepared by accurately weighing 10 mg of each standard on a closed electronic microbalance (Sartorius, Germany) and dissolving them separately in 10 mL of methanol. Calibration standard and quality control (QC) samples in plasma were prepared by adding corresponding working solutions with drug-free rat plasma. A volume of 10 mL of appropriate diluted stock solutions of mixture of drugs (DMF, MMF) at different concentrations and 10 mL of IS (IMP) at a fixed concentration were spiked into 100 mL of drug-free rat plasma to yield final concentrations of calibration samples 50,100,200,400,500,800,1000 and 2500 ng/mL for DMF and 10,25,50,100,200,300,400 and 500 ng/mL for MMF. The final concentration of IS (IMP) was 100 ng/mL. Similarly, QC samples were prepared at four concentration levels LLOQ, LQC, MQC and

HQC for DMF and MMF. The range of the calibration curves were 50–2500 ng/mL for DMF and 10–500 ng/mL for MMF.

Sample preparation

Rat plasma (50 µL) and 10 µL of IS working solution were individually transferred into a 1.5-mL microcentrifuge tube to which acetonitrile (250 µL) was added, and the solution was mixed to precipitate protein. The tubes were vortex mixed for 20 s and centrifuged at 10000 rpm for 3 min. The resulting supernatant (100 µL) was transferred into a 200 µL insert and 50 µL aliquot was analyzed by LC–MS.

Method Validation

Specificity

To determine whether there were endogenous compounds in the sample capable of producing a signal in the same retention time (tR) window, three different lots of blank plasma, blank plasma fortified with IS, and that fortified with 50 ng/mL DMF and 10 ng/mL MMF (limit of quantification, LOQ), were analyzed to determine the specificity of the method. The MRM chromatograms of MMF and DMF are represented in Fig. 2.

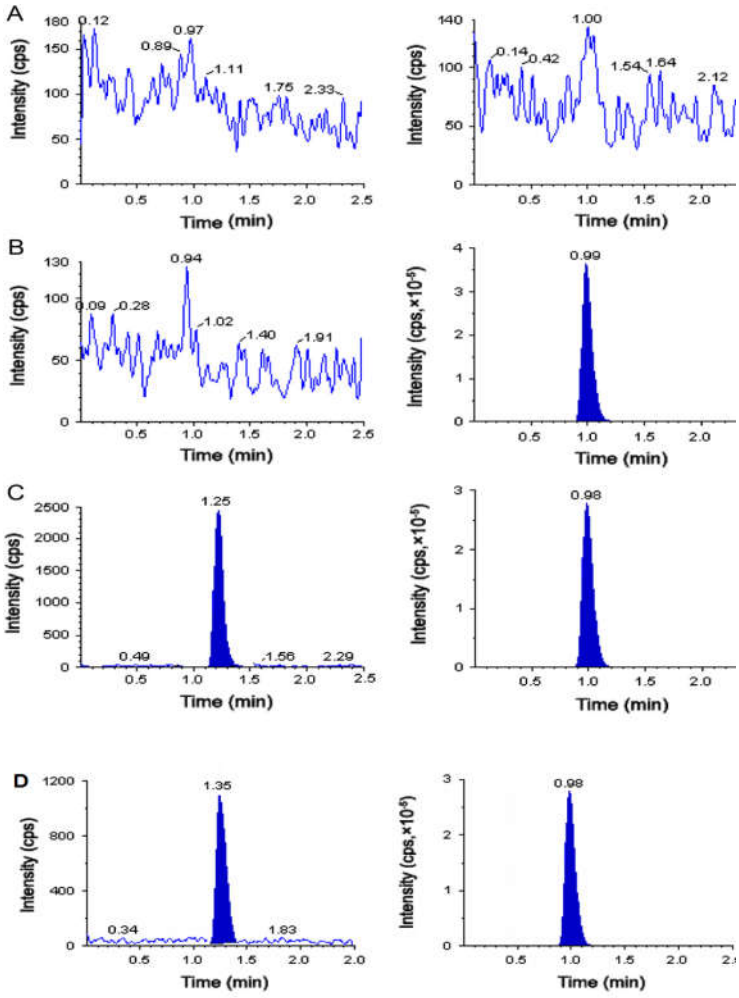

Figure 2: Typical MRM chromatograms of MMF & DMF (leftpanel) and IS (rightpanel) in rat blank plasma (A), and rat plasma spiked with IS (B), a LLOQ MMF sample along with IS (C) and LLOQ DMF sample along with IS (D).

Linearity

Linearity was evaluated for analyte concentration range of 20–2500 ng/mL for DMF and 10-500 ng/mL for MMF. Different weighting factors ($1/x$, $1/x2$, and none) were compared for best fit, based on the criteria that the deviation of the low LOQ from nominal concentration was < 20% and deviation of standards other than low LOQ was < 15% from nominal concentration.

Intra- and inter-day precision and accuracy

For evaluation of intraday precision and accuracy, three sets of six replicate plasma samples containing DMF and MMF at low, medium, and high QC levels were analyzed on the same day. As for inter-day precision and accuracy, three sets of two duplicate plasma samples containing the analytes at the same low, medium, and high concentrations were analyzed for three consecutive days.

Matrix effect

Matrix effect on suppression or enhancement of ionization of MMF was evaluated in three replicates at low, medium, and high concentrations of QC levels. Acetonitrile (250 µL) was added to blank plasma (50 µL) to precipitate proteins in the sample. The tubes were vortex mixed for 20 s prior to centrifugation (10000 rpm) for 3 min. An aliquot (10µL) of the MMF standard from each of the three concentrations was added to the tubes. Water instead of plasma was used to compare matrix effect contributed by plasma. For this purpose, an equal volume of water was used and treated as described for plasma. The same volume (10 µL) of MMF standard solutions as of water was added to the tubes, treated as described and then analyzed.

Stability of DMF and MMF in rat plasma

DMF at three different concentrations (100, 600, and 2000 ng/mL) and MMF at three different concentrations (30, 100, and 400 ng/mL) in rat plasma were prepared and stored at different temperature conditions (room temperature, 4°C, –20°C, and –70°C) to evaluate stability under these conditions. To assess stability of the analytes at room temperature (25°C), the plasma samples (5 mL each) in plastic test tubes (15 mL) were allowed to remain on the laboratory bench-top for 24 hrs, prepared and analyzed. For assessment of the stability for short-term storage (4°C), the plasma samples were stored at 4°C for a maximum of 10 days; the samples were analyzed after 10 days. For long-term storage, plasma samples were stored at –20°C and –70°C for a maximum of 4 weeks; the analytes were subsequently analyzed at 14, 21, and 28 days following storage. The mean concentrations of DMF and MMF from triplicate samples at each temperature and time point were determined. Evaluation of stability of DMF and MMF for long-term storage is particularly important to know because research samples are usually stored for a longer period of time.

3. Results and Discussion

Quantification and method validation

For quantification of DMF and MMF, sensitive MRM scan was employed using IMP as IS in rat plasma. Enhanced product ion spectra of DMF (Fig. 3A) and MMF (Fig. 3B) show the protonated molecules of m/z 145 → 115 and 131 → 115 respectively. The retention times of DMF, MMF and IS were 1.25, 1.35 and 0.99 min, respectively. Thus, three analytes were distinctly resolved from one another by the present LC method. It should be noted that interfering peaks from endogenous compounds were not observed at the same tR windows of both DMF and MMF. Calibration for the quantification of DMF and MMF was performed using the ratio of peak area of the analyte to that of IS. The ratio of peak area of the analyte to that of IS was proportional to analyte concentration from 50 - 2500 ng/mL for DMF and 10-500 ng/mL for MMF. A linear regression model was used to describe the regression relationship, and $1/x$ was the best linear fit of the calibration curve ($r2$ = 0.9999). The limit of detections (LOD; 25 ng/mL for DMF and 5 ng/mL for MMF) were defined as the lowest concentration of analytes that are spiked into plasma and resulted in MRM signal that was 3 times greater than noise. Limit of quantification (LOQ) was defined as the lowest concentration in the calibration curve that was measured with acceptable accuracy (within ± 20% of the theoretical value) and precision [coefficient of variation (CV) less than ± 20%] and the LOQ was 50 ng/mL for DMF and

5 ng/mL for MMF. The precision of the assay was expressed as the CV%, which was calculated as percent of the standard deviation divided by the mean of observed concentrations. The results of intra- and inter- day accuracy and precision are listed in Table 1. The results indicated that the method was accurate with excellent accuracy range of 93.3–102.68%, and the CV was within 10%. Matrix effect is a special phenomenon associated with LC–MS determination of drugs from biological fluids such as plasma and other matrices. Endogenous components extracted from plasma may suppress or enhance ionization of the analytes in electrospray source if they co-elute with the analytes. It is for this reason that matrix effect was evaluated under the experimental conditions used in this study. As shown in Table 2, the average ion suppression or enhancement for DMF and MMF at low QC and high QC levels was < 11%, suggesting that matrix effect on the analysis was negligible. The stability results (Table 3) showed that DMF and MMF spiked into rat plasma was stable for 24 h at ambient temperature, for 10 days at 4°C, and for 4 weeks at –20 and –70°C. Stability of the analyte in the sample is of crucial importance to the validity of the split sample program. Thus, proper storage of all samples is very important to obtaining reliable results and their interpretation in analysis.

Table 1: **Intra-day and Inter-day variation for DMF and MMF in six replicates (n=6) at each concentration**

Analyte	Nominal concentration (ng/mL)	Intra-day		Inter-day	
		% Recovery	% RSD	% Recovery	% RSD
DMF	50 (LLOQ QC)	97.83	7.035	101.49	7.192
	100 (Low QC)	99.31	4.598	93.29	4.797
	600 (Mid QC)	99.69	4.221	99.49	2.658
	2000 (High QC)	101.20	7.009	96.97	4.984
MMF	10 (LLOQ QC)	102.68	7.302	101.52	3.850
	30 (Low QC)	98.60	9.270	95.46	7.775
	100 (Mid QC)	100.622	3.126	98.80	3.535
	400 (High QC)	101.688	2.988	98.99	2.974

Table 2: Matrix effect results

Analyte	DMF				MMF			
	LQC		HQC		LQC		HQC	
	MF	ISNMF	MF	ISNMF	MF	ISNMF	MF	ISNMF
Lot 1	1.0602	0.9153	1.1295	1.0119	1.0526	0.9130	1.0708	0.9589
Lot 2	1.0381	0.9153	1.0824	0.9783	1.0745	0.9783	1.0170	0.9189
Lot 3	1.1619	1.1186	1.1007	0.9257	1.0442	0.9783	1.0985	0.9236
Lot 4	1.0471	0.9153	1.1467	0.9856	1.0698	0.9130	1.0478	0.9003
Lot 5	1.0855	0.9153	1.1117	0.9949	1.0142	0.8478	1.0916	0.9764
Lot 6	1.2419	1.1186	1.1421	1.0187	1.0331	0.9130	1.0474	0.9340
Mean		0.9831		0.9858		0.9239		0.9354
% CV		10.7		3.4		5.3		3.0

MF, Matrix Factor; ISNMF: IS Normalized Matrix Factor

Table 3: Stability studies of Dimethyl fumarate (DMF) and Monomethyl fumarate (MMF)

Analyte	Level	Mean accuracy / RSD (n=6)			
		Room Temperature (24h RT)	4°C	-20°C	-70°C
DMF	Low QC High QC	100.43 / 2.79 94.17 / 5.01	94.83 / 6.14 99.76 / 7.17	102.44 / 9.11 99.32 / 6.22	94.44 / 7.31 98.58 / 3.47
MMF	Low QC High QC	101.78 / 6.67 102.52 / 4.21	101.03 / 8.43 100.36 / 3.44	100.19 / 6.95 99.61 / 4.98	101.03 / 4.42 99.49 / 4.14

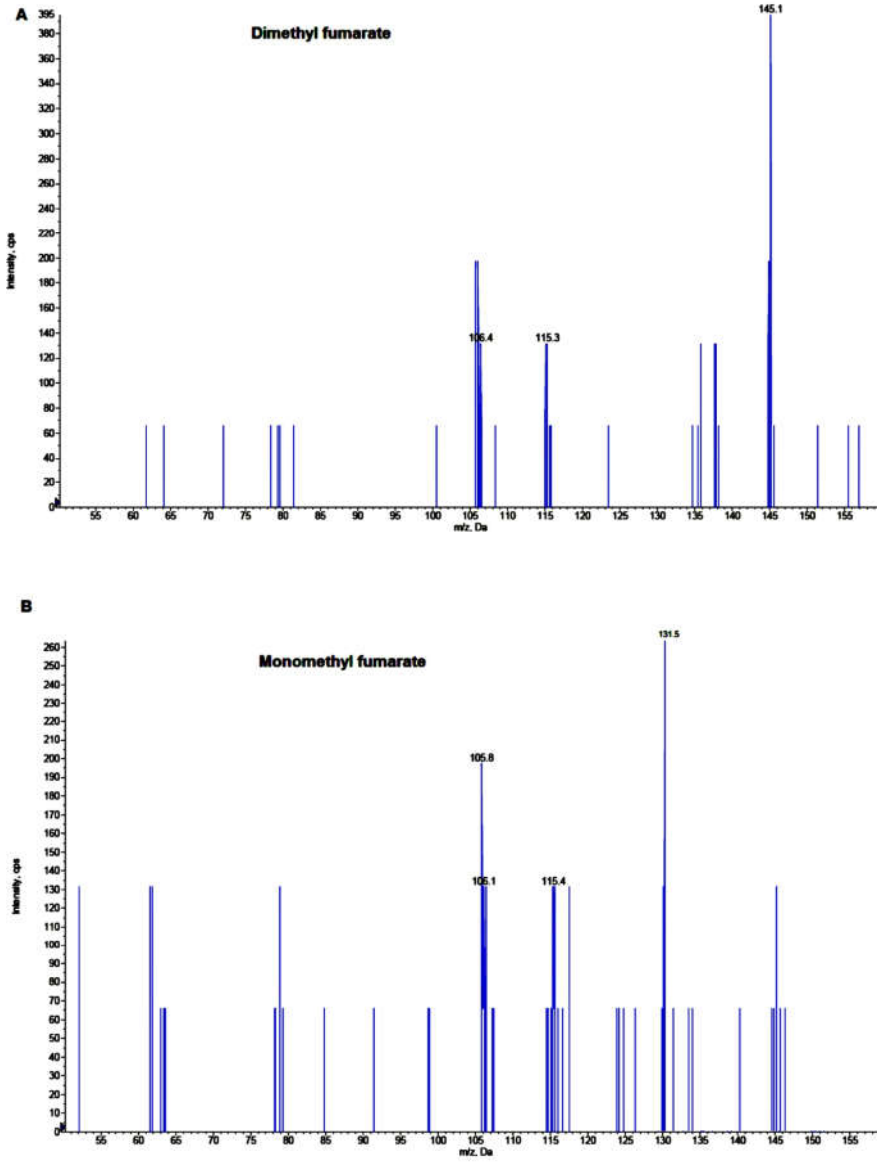

Figure 3: Product ion spectra of DMF and MMF

Estimation of measurement uncertainty

Measurement uncertainty (MU) is a parameter that describes the range of values which the measurand can be declared to lie within a specified level of confidence[13,15,16] because any measurement does not have a fixed known value. Measurement uncertainty can be estimated using laboratory quality control samples (LQCS) as previously demonstrated. In this study, MU was estimated using 25 QCS. As shown in the uncertainty budget (Table 4), standard uncertainty value was 4.2 with 24 degrees of freedom. The MU estimation for DMF at 95% confidence interval with a coverage factor of k = 2 was 8.6%. Thus, an estimated quantitative value of 400 ng/mL, DMF, as an example, would be reported as 400 ± 400 × 8.6% ng/mL (400 ± 34.4 ng/mL). Thus, the true value of DMF in the example analyzed lies between 365.6 and 434.4 ng/mL. Measurement uncertainty should only be reported at the request of the client in cases involving a commercial or contract laboratory.

Table 4: Estimation of Measurement Uncertainty of DMF and MMF

Parameter	Value units (%)	Advisor	Standard Uncertainty	Degrees of Freedom (n-1)
U_1 (Combined Uncertainty)	4.3% (DMF) 5.8 % (MMF)	$\sqrt{U_1^2}$ = 4.3 $\sqrt{U_1^2}$ = 5.8	4.3	24
Expanded Uncertainty (k= 2.0) n= 25	-	4.3 x 2.0 = 8.6 (DMF) 5.8 x 2.0 = 11.6 (MMF	5.8	24

Application of the method to pharmacokinetic study

In order to verify the sensitivity and selectivity of the developed method in a real-time situation, the developed LC-MS method was successfully applied to a pharmacokinetic study by administration of DMF as single solution to six male wistar rats by oral route using BD syringe attached with oral gavage needle (size 18) at the dose of 3 mg/kg body weight. Approximately, 0.2 mL of blood samples from each anesthetized (isoflurane) rat at predetermined time intervals was collected using a capillary tube into pre-labeled eppendorf tubes containing 10% of K2EDTA anticoagulant (20 µL). The time intervals for the sample collection were 0 (predose), 0.5, 1, 2, 4, 6, 8, 12 and 24 h (postdose). The total blood volume collected from each rat was approximately 1.7 to 1.9 mL which does not exceed the maximal recommended blood volume of 20% (2.0 mL for a 200 g body weight rat). Plasma was obtained by centrifuging blood samples at 4,000 rpm for 10 min. The obtained plasma samples were transferred into pre-labeled micro centrifuge tubes and stored at −50°C. All the samples were analyzed by the developed method and the mean plasma concentrations vs time profile of monomethyl fumarate is shown in Fig 4.

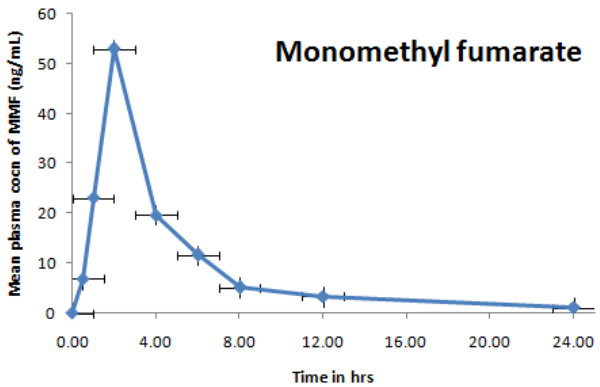

Figure 4: Mean plasma concentration–time profiles of MMF in rats

4. Conclusions

A highly selective and sensitive LC–MS method for screening, confirmation and quantification of DMF and MMF in rat plasma was developed and validated. For quantification, good linearity, accuracy, and precision were achieved. Stabilities of DMF and MMF at various storage conditions were determined. Measurement uncertainty was also estimated for this method. Confirmation of the presence of MMF in test samples was based upon the presence of MRM response within the correct tR window, qualitative match between EPI spectrum obtained for the unknown test sample and that of the corresponding reference standard in the library and product ion intensity ratios. The method is capable of providing defensible evidence for identification of DMF and MMF in rat plasma. The method has also been successfully used in the analysis of MMF for pharmacokinetic studies in rats. The method is fast, sensitive, selective, and reliably reproducible. As DMF immediately converts to MMF at in vivo conditions, this method is highly useful for the simultaneous determination of DMF and its major active metabolite MMF in clinical sample analysis of Phase III.

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